Functionalisation of free amino groups of lysine dendrigraft (DGL) polymers

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ABSTRACT

Lysine dendrigraft polymers (DGL) are promising candidates as carriers for targeted drug/gene delivery and bio-imaging, as such deserving extensive chemical functionalisation studies. We describe here examples of complete grafting of DGL amino groups by various substituents: hydrophobic amino acids (Ala, Val, Leu), dicarboxylic acids (succinic, Asp), guanidyl and saccharides (Gal), by means of straightforward coupling reactions, thus opening to versatile tuning of DGL properties (hydrophobicity, nucleophilicity, electric charge...). DGL functionalisation by lactose (when carried out so as to avoid borate ester formation between sugars) however yields partially grafted DGL: besides, partial grafting can be achieved by tuning the reagent/DGL stoichiometric ratio.

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The recently described expeditious preparation of lysine dendrigraft polymers (DGL) by our group,1 opened a breakthrough towards the availability of low-cost dendrimeric macromolecules, with numerous potential applications in, for example, health or life sciences. DGL present physicochemical properties very similar to those of dendrimers, for example, the ratio of viscosity to hydrodynamic radius,2 and turn out to be non immunogenic.3 Additionally, owing to a less crowded structure they are able to carry molecular objects within their dendritic framework. For instance, native DGL were recently used as nanosized magnetic resonance imaging contrast agents (after mixing with Gd4+ complexes),4 for gene delivery through the blood–brain barrier5 or for transport through liposomal and cellular membranes6 demonstrating that this new class of macromolecules might be used in large majority of applications developed for dendrimers such as gene therapy, drug delivery and bio-imaging.

Dendrigraft polymers address one of the main disadvantages of dendrimers, namely their tedious, stepwise synthesis requiring repeated synthesis and purification cycles. For example, 12 steps are necessary to prepare the 4th generation of polyamidoamine dendrimer (G5-PAMAM) bearing 128 primary amino end-groups.7 Alternatively, the use of inexpensive monomers and preparation techniques allows the preparation of lysine dendrigraft polymers bearing a high number of end-groups through a much lower number of polymerisation steps—for example, the 3rd generation of DGL (G3, bearing ca. 120 primary amino end-groups) is prepared within a mere three polymerisation/deprotection cycles.

However the polycationic character of DGL (because of its pending primary amines) is unsuitable to many applications; more generally, the access to a wide spectrum of functional end-groups is necessary to address the requirements of various dendrimer applications, for example, control of the toxicity,8,9 target recognition,10,11 hydrogel formation,12 bio-imaging and potentially a combination of the latter.13 This strongly raises the need of DGL amino end-group functionalisation by various compounds to modify, for example, its electric charge, acidity, hydrophobicity, or to introduce specifically reacting groups, by ways of straightforward, efficient and flexible synthetic methods, with the further need of tuning the functionalisation ratio and/or of mixing different functional groups in a controlled manner, while tolerating the presence of various functional entities (drugs, imaging agents...).14

In a preceding paper15 we addressed the issue of DGL core-functionalisation, by preparing a bifunctional initiator to be used in the DGL synthesis process. In this Letter, we describe the preliminary results of various amine functionalisation of DGL (acids, amino acids, guanidyl and sugars), exemplifying on the 2nd generation G2 (comb-like topology, ca. 50 Lys units: Mn = 6150 without counter-ions, polydispersity index PDI = 1.38).

According to the functional group to be grafted, well-known, efficient and robust synthetic procedures were used, which are summarised on Scheme 1. In all cases described here (except with lactose, see below), the use of a sufficient excess of reagent resulted in complete grafting of the DGL amino groups16 (both x and ε; reagent stoichiometries were calculated assuming the native DGL to bear one primary amino group per Lys residue). The grafted DGLs were purified by either precipitation or preparative size exclusion chromatography (SEC).17

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the DGL with amino acids is a way to alter the alkalinity of DGL G2(Y) in 91% yield, however without regioselectivity between α and γ carboxy groups of Asp; N-Tfa protecting groups were finally deprotected by treatment with ammonia in water/methanol.21 In both cases the final DGL conjugates were separated by preparative SEC in aqueous eluent. While the DGL-succinic conjugate features an inversion of the DGL electric charge (all free amino groups being replaced by carboxy groups), the DGL G2(Asp) conjugate features a polyzwitterionic macromolecule.

**Guaniylation** [D] of DGL amino groups both increases the basicity and suppresses their nucleophilicity. Guaniylated DGL G2(guan) was obtained in quantitative yield through the classical procedure by Bernatowicz et al.22 by reacting the primary amino groups of the DGL with 1H-pyrazole-1-carboxamidine hydrochloride (PCH) in aqueous NaHCO3—this classical method actually ensured an easier work-up than the method published by Paleos24 (who reacted DGL and PCH in DMF in the presence of DIEA). Although there is no additional 13C signal after DGL guanidylation (the quaternary 13C at 173 ppm is actually difficult to quantify), the guaniylation extent can be assessed from the amount of downfield shifted He resonances from 2.8 to 2.9 ppm (free ε-amino groups) to 3.1 ppm (ε-guanidyl groups); a similar 13C NMR downfield shift of Ce occurs from 39 ppm (free ε-amino groups) to 41 ppm (ε-guanidyl groups). Such quantification must however take into account the overlapping resonances of Nε-branched Lys residues (intrinsic to DGL structure and representing ca. 17% of total Lys residues in G2) at δce = 3.1–3.2 ppm (Hε, overlapping ε-guanidyl groups) and δce = 39 ppm (Ce, overlapping ε-amino groups).

**Grafting DGL with sugars [E–F] opens to cell/target-recognition and immunologic applications such as vaccines:** in this scope
Baigude et al. described the grafting of mono/oligo saccharides on PAMAM, lysine and ornithine dendrimers using the classical reductive amination reaction with borane/pyridine complex in borate buffer. Adapting Baigude’s protocol, an excess of galactose was reacted with DGL G2, the product then being acidified to pH 2 prior to preparative SEC separation in acidic eluent. This afforded the G2 Gal1 adduct in 77% yield with ca. 1.1 Gal units per Lys unit according to 1H NMR spectra on the basis of integration of signals in the 3.0–4.6 ppm range (all Gal + Lys Hα/c resonances) compared to Hβ of Lys at 1.2–1.8 ppm (at max. 2 Gal units could in theory be grafted onto each primary amino group). Lactose was grafted to DGL G2 by the classical NaBH4/CN mediated reductive amination, followed as above by acidic treatment at pH 4.5 prior to separation by preparative SEC, thus affording the grafted product G2 Lac0.33 in 80% yield however bearing only 0.33 lactose unit per Lys residue. Under the same conditions a fourfold excess of lactose did not improve the grafting extent, the latter was assessed by 1H NMR by the integration of Hβ = 1.2–2.0 ppm) versus anomeric H of lactose at 4.6 ppm (or equivalently vs the integration of all Lac signals in the 2.4–4.6 ppm range after deduction of contributions from Lys Hα and Hβ). Investigation is in progress to improve lactose grafting (limitations being possibly due to reactant bulkiness or premature imine hydrolysis prior to reduction).

While Baigude et al. stated that reducing amination allows the coupling of two (oligo)saccharide units per primary amino group, we however observed that without acidification prior to SEC separation (prior to dialysis as in Baigude’s works), additional saccharide units remain ‘overgrafted’ to the macromolecule through (intersaccharide) boric ester bonds—such work-up indeed affords the dialysed G2 Gal polymer containing ca. 2 Gal units per amino group and substantial amounts of borate esters. Such saccharide ‘overgrafting’ is acid-labile however, what may turn out to be unsuitable to certain applications.

Grafting of higher DGL generations: Although the GGL G2 has formally comb-like polylysine structure, the side chains of Lys residues make its actual structure rather close to a true branched polymer. In fact, preliminary results on higher DGL generations show qualitatively identical grafting, as exemplified with the grafting of DGL generation G4 (dendrigraft structure of ca. 365 Lys units: 1M = 46,700 without counter-ions, Pl = 1.36) with 2 equiv of succinic anhydride (same protocol as for G2) which afforded the completely grafted DGL G4 succ with 86% yield prior to preparative SEC separation.

Tuning the DGL grafting extent: Various degrees of grafting can be obtained by using a default reagent/DGL stoichiometric ratio. For instance, repeating the Bernatowicz method as above with a default PCH/DGL stoichiometry (0.1–0.5 equiv), reproducibly afforded partially guanylated DGL G2 guanex exhibiting identical characterisation as those described by Paleos et al. The grafting of G2 G2 with 0.3 or 0.5 equiv of lactose (relatively to Lys monomer units) also yielded grafted DGL G2 Lacx with lower grafting extents of 12% and 20%, respectively.

In summary, and in complement to the recently reported ‘ante-synthesis’ core functionalisation of DGLs, we show here (by exemplification on DGL G2) that ‘post-synthesis’ DGL functionalisation by hydrophobic amino acids, dicarboxylic acids, guanidyl and sugars, can be achieved using efficient and simple one-pot reactions with an easy work-up. Such new, biocompatible DGL bearing multiple functional groups, are promising candidates for the attachment of drugs, immunising or imaging agents. Further functionalisation methods currently under investigation, especially to address the grafting of bulky groups, as well as the pluri-functionalisation of the DGL through orthogonal grafting will be described in forthcoming papers.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.03.082.

References and notes

16. DGL G2 and G4 were prepared according to Collet et al. (Ref. 1) and used without further counter-ion exchange, reactant weights calculated assuming all lysine units in the DGL to be trithioacetic acid salts (Mw = 242 per monomer unit); reactant equivalents expressed versus monomer units.
17. In all examples preparative size exclusion chromatography (SEC) separations were carried out using Sephacryl® C 25 resin as the stationary phase, on an AKTA Purifier 100 system (General Electric) equipped with an UV-900 detector (elution monitored at λ = 210 nm).
18. Experimental procedure for G2(Ala). In a 25-mL flask fitted with a magnetic stirrer and stoppered with a silicon cap, DGL G2 (1000 mg 4.1 mol, 1.0 equiv) and Boc-Ala (1163 mg, 6.14 mmol, 1.5 equiv) were suspended in 5 mL DMF, to which was added DIEA (4.3 mL, 24.6 mmol, 6 equiv). The mixture was chilled to 0°C (ice bath), added with BOP (2994 mg, 6.77 mmol, 1.3 equiv), stirred at 0°C for 1 h then allowed to stir at rt for 3 h (monitoring: TLC/ninhydrin). DIEA was distilled off in vacuo, the mixture was precipitated in 300 mL of stirred aq K2CO3 (5% w/v). The precipitates were collected by centrifugation, washed 4 times with ether to remove TFA, then suspended in TFA (2 mL) then reacted at rt for 30 min. The resulting solution was poured in 50 mL cold (0°C) ether, to which were added 20 mL water. The aqueous phase was washed 4 times with ether to remove TFA, concentrated in vacuo to remove remaining ether, then freeze-dried to afford 1200 mg (3.83 mol, 93% yield) of G2(Ala). 1H NMR (D2O, 300 MHz) δ = 1.05 (2H, br, H2 of Lys); 1.18 (3H, d, J = 7.3 Hz, Hβ of Ala); 1.20 (2H, br, H6 of Lys); 1.24 (2H, H5 of Lys); 2.88 (2H, br, Hc of Lys); 3.71 (1H, br, g, J = 7.3 Hz, Hα of Ala); 3.83–4.08 (1H, br, Hα of Lys). G2 Val and G2 Leu were prepared on the 100 mg scale using the same procedure.
19. Experimental procedure for G2 succ. To a solution of DGL G2 (200 mg, 0.83 mmol monomer units) in 15 mL DMF placed in a 25-mL flask, was added succinic anhydride (167 mg, 1.67 mmol); the pH of the mixture was then adjusted to 8 with triethylamine. The mixture was stirred overnight at rt, then separated by SEC (elucent: 0.1 M aq NH4HCO3) and freeze-dried to afford the product as a white solid (240 mg, 0.70 mmol, 85% yield). 1H NMR (D2O, 200 MHz, 400 MHz).
Experimental procedure for 21. Lapidus, M.; Sweeney, M. 20
Experimental procedure for 22. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. 23

Added N-trifluoroacetyl-L-aspartic anhydride (471 mg, 2.23 mmol, 2 equiv) then triethylamine (466 µL, 3.35 mmol). The mixture was stirred at room temperature for 2 days, then the DMF was distilled off under vacuum (rotavapor). The residue was suspended in 40 mL of an NH₃/MeOH/H₂O (5.7 N (16% w/v) aqueous ammonia/Methanol) (1/4 v/v) then stirred overnight at 50 °C. The mixture was then concentrated in vacuo to 1/3 volume (to remove excess ammonia and methanol), then separated by SEC (eluent: 0.1 M aq NH₄HCO₃) and freeze-dried to afford the polymer G2/Asp) as a white solid (356 mg, 0.76 mmol monomer units, yield 91%). 1H NMR (D₂O, 300 MHz) ω = 1.22–1.61 (6H, m, H–H–H–H of Lys); 2.50–2.77 (2H, m, H₆ of Asp); 3.02 (2H, m, H₄ of Lys); 3.81 (1H, m, Hₑ of Asp); 4.04 (1H, m, Hₑ of Asp); 4.11 (1H, m, Hₓ of Lys).

To a solution of DGL G2 (100 mg, 0.42 mmol of Lys units, yield 97%) NMR spectra were fully consistent with those described by Baigude et al. (Refs.26,27). Reaction extent.

Nounesis, G.; Paleos, C. M. 22. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. J. Org. Chem. 1992, 57, 2497–2502. 23. Experimental procedure for G2/guan. In a 25-mL flask, DGL G2 (100 mg, 0.41 mmol monomer units), PCH (82 mg, 0.56 mmol) and Na₂CO₃ (30 mg, 0.28 mmol) were dissolved in 2 mL water. The mixture was stirred at rt for 3 days, then separated by SEC (eluent: 0.1 M aq NH₄HCO₃) and freeze-dried to afford the polymer G2/guan) as a white solid (116 mg, 0.40 mmol monomer units, yield 97%). NMR spectra were fully consistent with those described by Paleos et al. (Refs. 6,24).


Experimental procedure for G2/Gal. In a 100-mL flask, we introduced DGL G2 (780 mg, 3.23 mmol of Lys units), α(+)-galactose (1.68 g; 9.34 mmol, 6 equiv) in 40 mL of 0.4 M borate buffer (pH 9) under stirring. To this mixture was added borane–pyridine complex (1.51 mL, 14.94 mmol), then the mixture was stirred at 50 °C for 7 days. The warm mixture was then poured with 5 mL glacial AcOH and 10 mL water and stirred for another 3 h. After concentration in vacuo, the reaction mixture was separated by SEC (eluente: NH₄HCO₃, 0.1 M) and freeze-dried to afford 950 mg of polymer G2/Gal1.1 bicarbonate salt as a white solid in 77% yield (on basis of Mₓ = 383.2 g mol⁻¹). 1H NMR (D₂O, 400 MHz) ω = 1.1–1.4 (2H, m, Hₓ-Hₓ); 1.5–1.8 (4H, m, Hₓ-Hₓ); 2.85–2.98 (1H, m); 2.95–3.12 (1H, m); 3.15–3.28 (0.8H, m); 3.30–3.55 (4.2H, m); 3.75–3.82 (1.1H, m); 4.00–4.25 (1.5H, m).

Experimental procedure for G2/Lac. DGL G2 (100 mg, 0.42 mmol of Lys units) and α(+)-lactose (150 mg, 0.44 mmol, 1 equiv) were dissolved in 2 mL of 0.2 M phosphate buffer (pH 7.0), to which was added NaBH₃CN (77 mg, 1.2 mmol, 3 equiv). The mixture was then stirred at rt for 2 h. After concentration in vacuo, the reaction mixture was then acidified to pH 2 with 0.1 M HCl then separated by SEC (eluente: NH₄HCO₃, 0.1 M), and freeze-dried to afford 110 mg of crude polymer G2/Lac0.33 bicarbonate salt as a white solid in 80% yield (on basis of Mₓ = 500 g mol⁻¹ by monomer unit for 33% grafted Lac). NMR spectra fully consistent with those described by Baigude et al. (Refs. 26,27). Reaction repeated with 50 mg of DGL G2 (0.21 mmol of Lys units), 300 mg of lactose (0.88 mmol, 4 equiv) and 77 mg of NaBH₃CN (1.2 mmol, 6 equiv) in 1 mL phosphate buffer, affording 50 mg of polymer G2/Lac0.33, same grafting extent.

Elemental analysis of dialysed polymer, found: B: 2.43; C: 33.58; H: 5.89; N: 2.63. 11B NMR spectra exhibit resonances at –10 and –13.5 ppm, attributed to mono- and bidentate esters, respectively.

Experimental procedure for partial Lac grafting: The same protocol as in Note 29 was applied with a different reactant/DGL stoichiometric ratio on the basis of 50 mg DGL G2 and either: (i) 75 mg of lactose, affording 40 mg of grafted polymer G2/Lac0.12; 76% yield on basis of estimated Mₓ = 255 g per monomer unit; or (ii) 25 mg of lactose, affording 37 mg of grafted polymer G2/Lac0.12; 78% yield on the basis of estimated Mₓ = 229 per monomer unit.